

***Bacillus licheniformis* penicillinase: Cleavages and attachment of lipid during cotranslational secretion**

(extracellular labeling/membrane-bound polysomes/membrane-bound penicillinase/penicillinase precursor/lipoproteins)

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ABSTRACT The penicillinase of *Bacillus licheniformis* is shown to be secreted cotranslationally. In extracts it was formed by membrane-associated but not by free polysomes; and after extracellular labeling of cells, followed by completion of the growing chains on polysomes *in vitro*, labeled penicillinase could be immunoprecipitated. This product contained electrophoretic peaks of M_r 36,000, 33,000, and 29,000, which correspond to previously reported forms of the enzyme. The M_r 36,000 form exhibits moderate hydrophobicity, as expected of a precursor with an NH_2 -terminal signal sequence for secretion. In addition, part of the M_r 33,000 fraction evidently contains a lipid: it is even more hydrophobic, and [$2\text{-}^3\text{H}$]glycerol was found to be incorporated into these molecules but not into the other forms of the enzyme. These findings renew the earlier, discarded suggestion that the M_r 33,000 membrane-bound penicillinase in the cells contains lipid. The incorporation of lipid and two different cleavages can evidently all occur during growth of the penicillinase chain. Moreover, the resulting terminal regions are all accessible to extracellular labeling on growing chains. Several additional, unidentified lipoproteins also incorporate lipid during chain growth.

The secretion of penicillinase by *Bacillus licheniformis* is unusual in involving a membrane-bound form (1), of M_r 33,000, as well as multiple extracellular forms, of M_r 29,000–33,000 (for variations in the M_r of the several penicillinase forms in different studies see ref. 2). Though the membrane penicillinase can be converted to exoenzyme, kinetic studies suggest that it is not an obligatory intermediate (3), and in extracts the conversion can be carried out by a protease (PR-protease) that is released from cells when they are converted to protoplasts (4, 5). The stable binding of M_r 33,000 molecules to membrane appeared to be accounted for by the finding of an NH_2 -terminal phosphatide (6, 7), but the presence of a lipid could not be confirmed (8) and has been considered doubtful by the original investigators (9).

Several other, more simply secreted, proteins have been shown to be synthesized as a precursor with an additional hydrophobic NH_2 -terminal signal segment (10–13), and they are secreted cotranslationally, as demonstrated by extracellular labeling of growing chains (14–17). The present experiments were undertaken to see whether penicillinase shares these features. Chain completion on membrane-associated polysomes did indeed form a larger precursor, of M_r ca. 36,000, along with M_r 33,000 and 29,000 forms; and in the cells the growing chains could be labeled extracellularly with a radioactive reagent. While this work was in progress, a M_r 36,000 form was also obtained by others, using different *in vitro* systems (9, 18).

The extra sequence on the M_r 36,000 form is evidently a hydrophobic signal peptide, because decylagarose retained these molecules more firmly than the M_r 29,000 or some of the M_r 33,000 molecules. Unexpectedly, other M_r 33,000 molecules were found to be even more hydrophobic. This finding renewed the possibility of a lipoprotein form, and this conclusion was

strongly supported by showing that the hydrophobic M_r 33,000 fraction, but not the others, has incorporated glycerol. It is of interest that this incorporation and cleavage can occur while the chains are growing and being secreted.

We have briefly reported the formation of the M_r 36,000 precursor and the incorporation of glycerol into M_r 33,000 molecules (14); we have not pursued the structure of the lipid. Other laboratories have now identified it, in penicillinase from cell membrane, as a glyceride attached to an NH_2 -terminal cysteine (19, 20).

MATERIALS AND METHODS

Preparation of Bacterial Cells and Fractions. *B. licheniformis* 749/C (kindly provided by J. O. Lampen), a constitutive hyperproducer of penicillinase (21), was grown to midlogarithmic phase in minimal medium A (22) supplemented with 0.4% glucose and 0.2% Casamino acids. Chloramphenicol (200 $\mu\text{g}/\text{ml}$) was added, the culture was poured over ice, and the cells were harvested. As previously described for *B. subtilis* (16), protoplasts were formed in buffer A [10 mM Tris-HCl, pH 7.6/50 mM KCl/10 mM $\text{Mg}(\text{OAc})_2$ /1 mM dithiothreitol] containing 25% sucrose and chloramphenicol at 200 $\mu\text{g}/\text{ml}$. The protoplasts were lysed osmotically and treated with DNase, and the membrane-bound polysomes and free polysomes were isolated by chromatography on Sepharose 2B (16). Derived polysomes were prepared from the membrane-polysome fraction by washing three times with buffer containing deoxycholate as described previously (15), except that 0.1% instead of 1% deoxycholate was used in order to minimize precipitation by Mg^{2+} , and 1.8 M sucrose in buffer A was used as a cushion to hold back any such precipitate from the pelleted polysomes. All steps in the fractionation were carried out at 0–4°C.

Extracellular Labeling of Nascent Chains. The extracellular labeling was carried out as described (16), with the following corrections and modifications. [^{125}I]iodosulfanilic acid (500 μCi at 2000 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels) was dried under reduced pressure and converted to the diazonium salt by incubation for 10–15 min at 0°C with 10 μl of 0.05 M NaNO_2 and 10 μl of 0.1 M HCl. The protoplasts from 1.2×10^{10} cells were prepared as described above, centrifuged, suspended in 50–100 μl of 10 mM NaH_2PO_4 (pH 7.5) with 25% sucrose and chloramphenicol at 200 $\mu\text{g}/\text{ml}$, and incubated with the diazonium salt mixture (final concentration 2–4 μM) for 15 min at 0°C with gentle shaking. The protoplasts were then centrifuged, washed with buffer A, and lysed osmotically, as described (16), and the polysomes were derived from complexes with membrane as in the preceding section.

Chain Completion. Membrane-associated or membrane-derived polysomes (1 A_{260} unit) were incubated at 37°C in a pro-

Abbreviation: DSA, diazosulfanilic acid.

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tein-synthesizing system (100 μ l) with S100 extract from *Escherichia coli* (15), in the presence of protease inhibitors (1 mM phenylmethylsulfonyl fluoride and 0.1 mM *o*-phenanthroline). After 30 min the mixture was chilled, Triton X-100 was added to 1% (to solubilize any membrane-bound or hydrophobic product), and the released chains were recovered in the supernate after sedimentation at $300,000 \times g$ for 3 hr.

Immunoprecipitation and Electrophoresis. To identify radioactively labeled penicillinase in the completed polypeptides, 100 μ l of the above supernate was added to 400 μ l of buffer containing 10 mM NaH_2PO_4 (pH 7.2), 0.14 M NaCl, and protease inhibitors as above. To this mixture was added 50 μ l of buffer containing 265 μ g of purified IgG antibodies to exopenicillinase, with 3 μ g of pure exopenicillinase as carrier. After incubation at 37°C for 3 hr the precipitates were collected, washed, dissolved in buffer containing 1% NaDodSO₄, boiled for 5 min, and examined by NaDodSO₄ polyacrylamide gel electrophoresis as described (15).

Reagents. Sepharose 2B was from Pharmacia. [³⁵S]Methionine (200–600 Ci/mmol), [2-³H]glycerol (9.5 Ci/mmol), and a kit for diazotizing [¹²⁵I]iodosulfanilic acid (2000 Ci/mmol) were from New England Nuclear. S100 extract from *E. coli* MRE600 was prepared as described (23). Pure M_r 29,000 exopenicillinase, penicillinase from membrane, and penicillinase-releasing protease from *B. licheniformis*, and antibodies to the exopenicillinase, were generously provided by J. O. Lampen. Sodium deoxycholate was from Schwarz/Mann. Other reagents were from commercial sources.

RESULTS

Synthesis on Membrane-Bound Polysomes. The penicillinase of *B. licheniformis* is evidently synthesized on membrane-associated polysomes, because per unit OD the membrane-polysome fraction synthesized 6 times as much penicillinase, and only half as much protein, as did the free polysome fraction (Table 1). The slight activity of the free fraction could be due in part to imperfect separation from membrane-polysome complexes (as suggested by its glycerol content: see Table 3 below). In addition, polysomes destined for attachment would be expected to remain free until their nascent chains were long enough to interact with membrane.

Extracellular Labeling of Nascent Chains. To demonstrate nascent chains traversing the plasma membrane, protein synthesis was blocked by chilling and addition of chloramphenicol, and the cells were converted to protoplasts, treated with [¹²⁵I]-labeled diazosulfanilic acid ([¹²⁵I]-DSA), and fractionated (see *Materials and Methods*). Little or no [¹²⁵I]-DSA had penetrated into the cells, because the free polysome fraction had only ca. 2% of the total label incorporated compared to 30% in the mem-

brane-polysome fraction; moreover, more than 80% of this 2% was released by treatment with both deoxycholate and puromycin (data not shown).

The polysomes from the membrane-polysome fraction, freed of membrane by washing three times with 0.1% deoxycholate, retained ca. 12% of the total label from [¹²⁵I]-DSA. As previously observed with *B. subtilis* (16), more than 75% of this label was on nascent chains, because it was released by treatment with 10 μ M Mg^{2+} or with puromycin, or by completing the chains. Moreover, chain completion doubled the average M_r of the labeled material (23,000, compared with 12,000 after release by puromycin), as previously observed with other extracellularly labeled chains (15).

Identification of Multiple Forms of Penicillinase. When the extracellularly labeled chains on polysomes were completed and the released products were separated from the ribosomes, antibodies to penicillinase precipitated about 10% of the released label. As Fig. 1 shows, this label was distributed about 60% in a M_r 29,000 peak and 30% in an overlapping M_r 33,000 peak, corresponding in M_r to the reference exo- and membrane-derived enzymes. In addition, about 10% was present in a larger peak at M_r 36,000, presumably a precursor.

To increase the labeling of the M_r 36,000 form relative to the other forms, we shifted from labeling with [¹²⁵I]-DSA to labeling by incorporation of [³⁵S]methionine during chain completion *in vitro*. The rationale was that short nascent chains would be more likely than longer chains to have remained unprocessed at the time of separation from membrane; they would attach less extracellular label; and they would incorporate more labeled amino acid during chain completion. Incorporation of methionine did indeed result in a higher ratio of labeled M_r 36,000 and 33,000 to 29,000 penicillinase (Fig. 2), compared with extracellular labeling (Fig. 1); other experiments showed an even

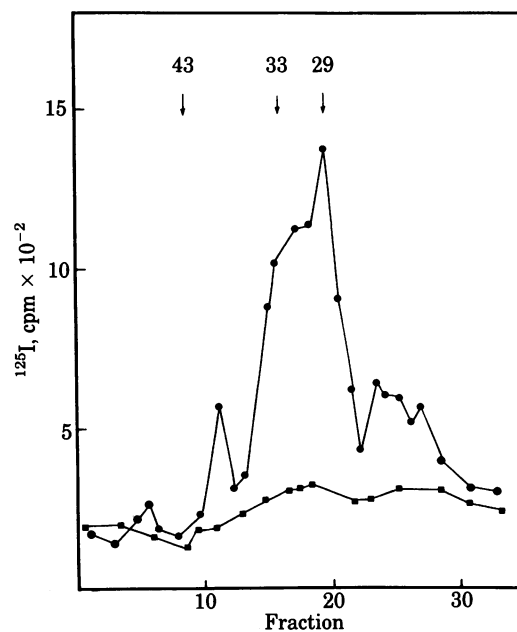


FIG. 1. Identification of penicillinase labeled with [¹²⁵I]-DSA. Protoplasts from 20 ml of culture were labeled with [¹²⁵I]-DSA and fractionated. One A_{260} unit of membrane-derived polysomes, containing ca. 25,000 cpm of [¹²⁵I], was incubated in a protein-synthesizing system with or without puromycin (200 μ g/ml). The released chains were separated from ribosomes, precipitated by IgG antibodies to penicillinase, and analyzed by gel electrophoresis. The M_r markers (indicated $\times 10^{-3}$) are: 43,000, alkaline phosphatase; 33,000, membrane penicillinase; 29,000, exopenicillinase. Negligible label was precipitated by normal serum. ●, Completed chains; ■, chains released by puromycin.

Table 1. Synthesis of [³⁵S]-labeled penicillinase by membrane-associated polysomes

| Protein | Free polysomes, cpm | Membrane-associated polysomes, cpm |
|---------------|---------------------|------------------------------------|
| Total protein | 523,000 | 275,000 |
| Penicillinase | 7,240 | 43,300 |

Free polysomes and membrane-associated polysomes of *B. licheniformis* (1.0 A_{260} unit) were allowed to complete their chains in the presence of [³⁵S]methionine. (About twice as much free as membrane-associated polysomes was obtained from the lysate.) In one aliquot total protein was precipitated by trichloroacetic acid; in another the products were incubated with antiserum to exopenicillinase (together with carrier penicillinase) and precipitated radioactivity was determined. Normal serum precipitated less than 0.1% of the total radioactivity, and this background was subtracted.

greater increase (see Table 2). This difference provides strong evidence for a precursor function of the M_r 36,000 fraction.

Cleavage by Protease. Treatment of the products of chain completion with pure penicillinase-releasing (PR) protease from *B. licheniformis* converted all the M_r 36,000 and 33,000 penicillinase molecules to 29,000 (data not shown). This finding is consistent with a precursor function for these molecules. Trypsin caused similar processing. Curiously, *E. coli* membrane preparations were inactive, though they can process precursors of *E. coli* alkaline phosphatase (13) and of diphtheria toxin (17).

Membrane-polysome preparations yielded completed penicillinase chains with much the same size distribution as those completed after removal of membrane. This finding contrasts with the need to eliminate membrane in order to obtain precursors of several other secreted bacterial enzymes.

Decylagarose Chromatography of Penicillinase. Decylagarose can be used to separate more hydrophobic from less hydrophobic proteins at various salt concentrations (24): for example, it retains a more hydrophobic precursor, but not the mature form, of *E. coli* alkaline phosphatase (13). We fractionated ^{35}S -labeled penicillinase in this way and examined the fractions for their M_r distribution. As Table 2 shows, the small exo form (M_r 29,000), and much of the M_r 33,000 form, passed through the column at 0.2 M NaCl, while the precursor form (M_r 36,000) did not elute under these conditions but did at 2 M NaCl. These results show that the M_r 36,000 form is more hydrophobic than the 29,000 or much of the 33,000 fraction, as expected if it has an additional signal sequence.

Unexpectedly, the M_r 33,000 product showed two distinct fractions, in various proportions in different experiments. In the experiment of Table 2, ca. 60% was eluted by 0.2 M NaCl, while the remainder was retained even at 5 M NaCl. This result suggested that the hydrophobic M_r 33,000 fraction carries a lipid.

Incorporation of Glycerol. To test for such attachment of a lipid, cells were grown for 30 min in the presence of $[2\text{-}^3\text{H}]\text{glycerol}$ and were fractionated. The polysomes were derived from complexes with membrane and the chains were completed. A small fraction of the total incorporated glycerol radioactivity (1% or less in various experiments) was recovered in the polysomes derived from membrane-polysome complexes. This label was evidently present in lipid, rather than in amino acids, because the free polysomes were negligibly labeled (Table 3). When the chains on the derived polysomes were completed about 1/10th of the radioactivity of the poly-

 Table 3. Distribution of incorporated $[2\text{-}^3\text{H}]\text{glycerol}$

| Fraction | cpm |
|---|---------|
| a, Derived polysomes | 240,000 |
| b, Free polysomes | 27,000 |
| c, Material released from derived polysomes by chain completion | 170,000 |
| d, Penicillinase immunoprecipitated from c | 23,000 |
| e, Penicillinase from d retained by decylagarose | 20,000 |

$[2\text{-}^3\text{H}]\text{Glycerol}$ (50 μCi) was added to a growing culture (25 ml) 30 min prior to harvesting the midlogarithmic-phase cells. Cells were lysed and fractionated, and chains were completed on the derived polysomes and immunoprecipitated. The immunoprecipitated penicillinase was dissolved and a portion was applied to decylagarose and tested for retention after elution by 5 M NaCl, as for Table 2. Normal serum precipitated less than 10% as much radioactivity as the specific antibodies.

somes could be precipitated by antibodies to penicillinase. Moreover, like the hydrophobic M_r 33,000 fraction labeled with methionine, the penicillinase labeled with glycerol was retained by decylagarose at 5 M NaCl (Table 3).

To compare more carefully the distribution of glycerol and that of protein among the several forms of penicillinase, polysomes from glycerol-labeled cells were allowed to complete their chains in the presence of $[^{35}\text{S}]\text{methionine}$. The glycerol label in the immunoprecipitated penicillinase was sharply limited to the M_r 33,000 fraction (Fig. 2). The label appears to be covalently linked, because it was not appreciably removed by washing three times with boiling 1% NaDodSO₄ (precipitating with trichloroacetic acid between washings), and it was still

 Table 2. Decylagarose chromatography of ^{35}S -labeled completed penicillinase chains

| Fraction | Penicillinase form, cpm | | |
|---|-------------------------|--------------|--------------|
| | M_r 36,000 | M_r 33,000 | M_r 29,000 |
| Total penicillinase | 32,400 | 28,800 | 40,800 |
| 0.2 M NaCl eluate | 1,410 | 16,800 | 37,800 |
| 2.0 M NaCl eluate | 27,600 | 700 | 1,050 |
| 5.0 M NaCl eluate | 500 | 750 | 1,000 |
| Radioactivity retained by decylagarose (calculated) | 1,830 | 10,550 | 950 |

Part of a redissolved ^{35}S -labeled penicillinase immunoprecipitate was analyzed by gel electrophoresis (top row). Another portion was dissolved in 0.1 ml of 0.1 M NaOH in 10 mM Tris-HCl, pH 8.0 (final pH 12.3), with 0.2 M NaCl, applied to a 0.5-ml decylagarose column, and successively eluted with a 2-ml portion of NaCl at each of the concentrations noted, in 10 mM Tris-HCl, pH 8.0. The eluates were collected, dialyzed against distilled H₂O, lyophilized, and analyzed by electrophoresis as in Fig. 1. The label retained by the decylagarose after the elutions was calculated, for each M_r peak, by subtracting the total radioactivity eluted from the amount deposited.

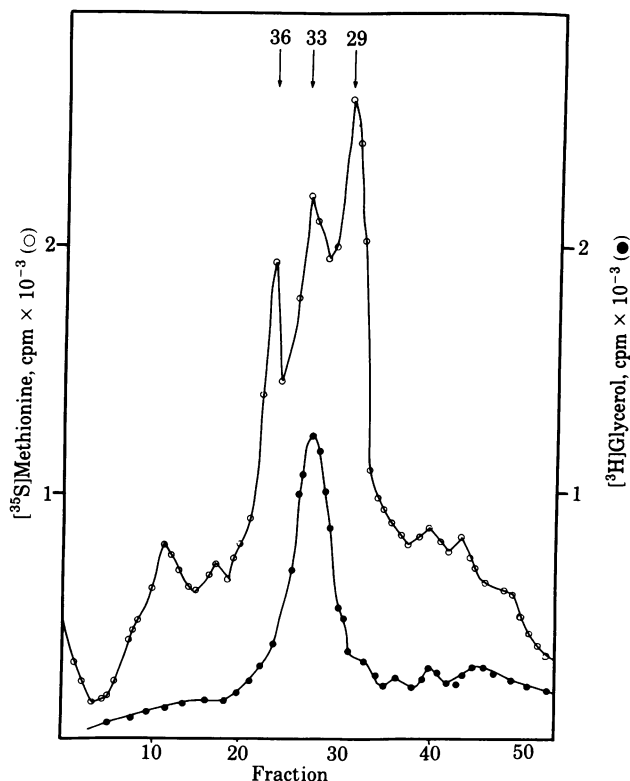


FIG. 2. Labeling of penicillinase with glycerol and with methionine. Derived polysomes, from cells labeled with $[2\text{-}^3\text{H}]\text{glycerol}$ as for Table 3, were allowed to complete their nascent chains in the presence of $[^{35}\text{S}]\text{methionine}$. The released chains were treated with antibodies to penicillinase and the precipitate was analyzed as in Fig. 1. Correction was made for crossover of ^{35}S cpm in ^3H (about 15%). ●, $[^3\text{H}]\text{Glycerol}$, incorporated *in vivo*; ○, $[^{35}\text{S}]\text{methionine}$, incorporated during chain completion *in vitro*.

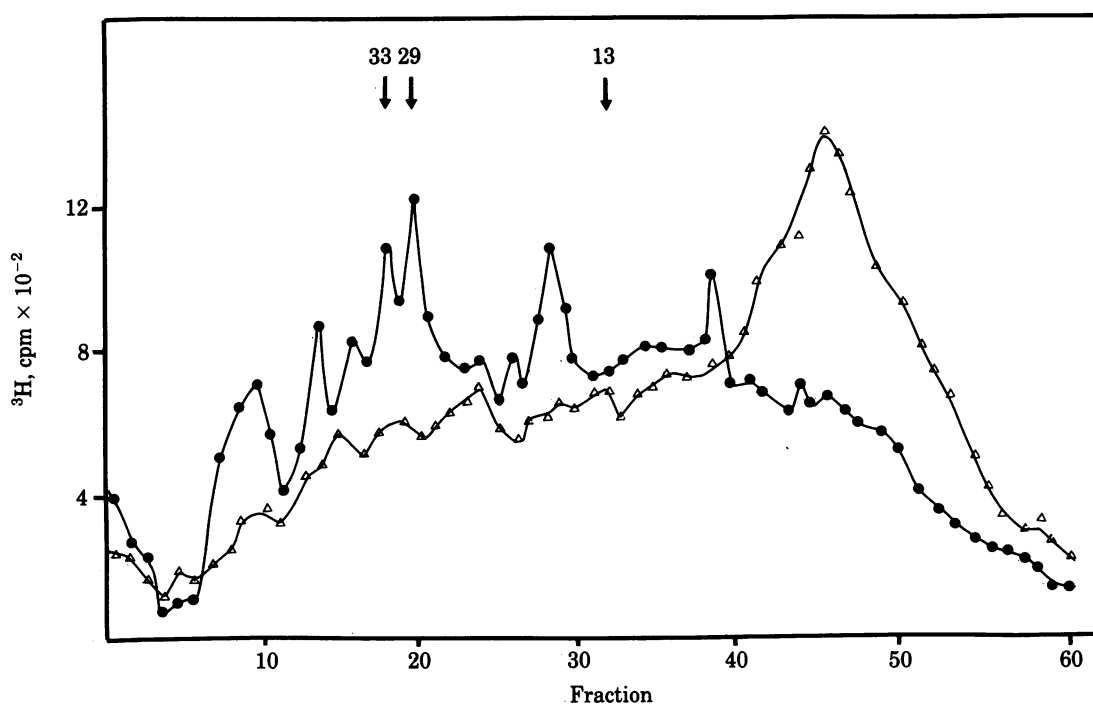


FIG. 3. Incorporation of $[2\text{-}^3\text{H}]\text{glycerol}$ into nascent chains of multiple lipoproteins. Derived polysomes, from cells labeled with $[2\text{-}^3\text{H}]\text{glycerol}$ as for Table 3, were allowed to complete their chains (●) or were treated with puromycin to release the nascent chains (Δ). Both sets of released chains were washed three times with boiling 1% NaDodSO₄ (precipitated in between by 5% trichloroacetic acid), and the samples were analyzed by electrophoresis.

present in the M_r 33,000 band after it had been redissolved and reelectrophoresed. Moreover, because the additional, hydrophobic sequence in the M_r 36,000 molecules would be expected to promote any noncovalent complexing with lipid, the presence of glycerol in the M_r 33,000 but not in the 36,000 fraction provides strong additional evidence for a covalent attachment. This finding also indicates that the attachment occurs during or after cleavage of the M_r 36,000 molecule, rather than via a stable M_r 36,000 protein-lipid intermediate.

The ratio of the two kinds of M_r 33,000 penicillinase in the completed chains was quite variable, and in some preparations no incorporation of $[^3\text{H}]\text{glycerol}$ could be detected. The presence of protease inhibitors (phenylmethylsulfonyl fluoride and *o*-phenanthroline) failed to ensure a regular recovery.

Multiple Lipoproteins Incorporating Glycerol During Chain Growth. As was noted in Table 3, the immunoprecipitated penicillinase contained only a small fraction of the glycerol found on the chains completed on polysomes (and retained after washing three times with boiling NaDodSO₄ solution). The fraction was quite variable. When the total released products of chain completion (on polysomes from glycerol-labeled cells) were examined by electrophoresis, eight or more bands were found to contain glycerol, some with at least as much as the penicillinase at M_r 33,000 (Fig. 3). These findings are in harmony with earlier evidence (25) for several unknown lipoproteins in the membrane of *B. licheniformis*. In addition, they show that the lipid is attached to these proteins during chain growth.

Fig. 3 further shows that these sharp peaks of $[2\text{-}^3\text{H}]\text{glycerol}$ did not appear until the chains were completed: when the incomplete chains were released by puromycin the radioactive glycerol had a much flatter electrophoretic distribution, and a much lower average M_r . These patterns are incompatible with an independent, overlapping distribution of contaminating membrane lipids and nascent or completed chains. Instead, much of the lipid, at least, is evidently associated with specific chains; and because the association is fixed long before the

chains are complete, it is probably covalent.

DISCUSSION

In the experiments reported here with *B. licheniformis*, as in earlier experiments with *B. subtilis*, *E. coli*, and *Corynebacterium diphtheriae* (15–17), peptide chains labeled extracellularly with ^{125}I -DSA included nascent chains growing on polysomes. Among the products of completion of these chains, penicillinase could be identified by immunoprecipitation, and it was formed only by membrane-associated and not significantly by free polysomes. It is evident that this enzyme is secreted cotranslationally.

On electrophoresis in NaDodSO₄/polyacrylamide gels the immunoprecipitated penicillinase appeared not only at M_r 29,000 and 33,000, which presumably correspond to an exoenzyme and to membrane-bound enzyme, but also at *ca.* M_r 36,000. This third form of the enzyme is evidently a precursor that carries an additional, hydrophobic sequence (Table 1). After this work was completed, molecules of this size were reported to be formed in a DNA-coupled system (18) and also in an mRNA-directed incorporation system (9); the latter product was shown to have an NH₂-terminal formylmethionine (9).

Decylagarose chromatography further revealed two forms in the M_r 33,000 penicillinase released from polysomes (Table 1), one hydrophilic and the other strongly hydrophobic. The hydrophilic form may be one of the larger exoenzymes reported by Lampen (2). The hydrophobic form evidently contains a lipid, because this fraction, but not the others, incorporated $[2\text{-}^3\text{H}]\text{glycerol}$ into its nascent chain in the cell (Fig. 2; Table 2).

The radioactivity in the hydrophobic M_r 33,000 fraction was retained after multiple washings with boiling NaDodSO₄ solution, and also after extraction of the M_r 33,000 band from the gel followed by a second electrophoresis. These findings strongly suggest a covalent linkage to the protein. Moreover, because the M_r 36,000 penicillinase differs from the M_r 33,000

by its content of an additional hydrophobic segment, which should promote any noncovalent complexing, the specific association of glycerol with the M_r 33,000 and not with the 36,000 form makes a covalent attachment seem virtually certain. Our hydrophobic M_r 33,000 penicillinase molecules are presumably identical with the membrane-bound M_r 33,000 penicillinase recovered from cells of the same organism, and the accompanying papers show that that material contains glyceride, attached by thioether linkage to an NH_2 -terminal cysteine (19, 20).

Among the products of chain completion on polysomes from glycerol-labeled cells, label appeared in a number of unidentified peaks, in addition to penicillinase. Not only was this label retained after several washings with boiling NaDodSO_4 solution, but it clearly had become associated with specific growing chains long before their completion, because release of these chains by puromycin yielded a preparation in which the label was distributed diffusely rather than in peaks, and with a much smaller average M_r value (Fig. 3). These findings strongly suggest that the several glycerol-labeled peaks are all lipoproteins, with lipid covalently attached to their NH_2 -terminal region.

It is of particular interest that the penicillinase lipoprotein, and also the unidentified lipoproteins, have incorporated lipid while the chains are still growing. In this process our findings do not indicate whether the M_r 36,000 precursor penicillinase is first cleaved to a hydrophilic M_r 33,000 product, to which lipid is then added, or whether the M_r 33,000 moiety is directly transferred from the precursor to a lipid, thus retaining an attachment to membrane. The lipid also might be attached first to the precursor, as has been observed for outer membrane lipoprotein of *E. coli* in a mutant blocked in processing (20, 26), or in cells inhibited with globomycin (27). However, we observed no labeling of the M_r 36,000 penicillinase peak with glycerol, and in *B. licheniformis* cells globomycin blocks incorporation of lipid into any form of penicillinase (19).

The incorporation of lipid into M_r 33,000 termini during chain growth clearly implies that the required cleavage of the M_r 36,000 precursor to 33,000 also occurs during chain growth. A similar cleavage of M_r 36,000 or 33,000 termini during chain growth would explain our recovery of M_r 29,000 completed chains. However, the observed multiple small cleavages of the NH_2 terminus of already secreted penicillinase in the medium (2) raise the possibility that the M_r 29,000 cleavage on our ribosome-attached chains has resulted from increased access to a protease during preparation or fractionation of the protoplasts. Two findings speak against such an artefact: the size distribution of the completed molecules was not significantly altered by (i) the presence of protease inhibitors throughout the preparation of polysomes from cells or (ii) chain completion on membrane-associated rather than on derived polysomes.

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